The Journal of Immunology

Characterization and Application of a Unique Panel of Monoclonal Antibodies Generated against Etanercept

Iris Detrez, Els Brouwers, Miet Peeters, Nick Geukens, Kurt de Vlam and Ann Gils

J Immunol 2016; 196:2879-2884; Prepublished online 3 February 2016;
doi: 10.4049/jimmunol.1502195
http://www.jimmunol.org/content/196/6/2879

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Characterization and Application of a Unique Panel of Monoclonal Antibodies Generated against Etanercept

Iris Detrez,* Els Brouwers,* Miet Peeters,* Nick Geukens,† Kurt de Vlam,‡ and Ann Gils*

The clinical response in ankylosing spondylitis (AS) patients treated with biologic agents can be influenced by pharmacokinetic variability among and within these patients. Therapeutic drug monitoring is seen as a valuable tool to improve patient care. The aim of this study was to generate a panel of mAbs toward etanercept (ETN) and to determine ETN and anti-ETN concentrations in AS patients. mAbs against ETN (MA-ETN) were generated using hybridoma technology. For quantification of ETN concentrations, a mAb-based TNF-coated ELISA and a mAb/mAb-based sandwich-type ELISA were developed. For evaluation of the anti-ETN Ab response, a bridging ELISA, as well as a functional cell-based assay, were constructed. Disease activity of the AS patients was measured with the AS Disease Activity Score (ASDAS). Active disease was defined as ASDAS ≥ 2.1. A total of 59 of 76 generated mAbs were ETN specific and were characterized further. Fifty-one mAbs revealed inhibitory properties in a cell-based assay. Analysis of serum concentrations of 21 ETN-treated AS patients with the TNF/MA-ETN68C5-HRP ELISA and the MA-ETN63C8/MA-ETN61C1-HRP ELISA revealed a good Pearson's *r* (+0.974) but a poor intraclass correlation coefficient (+0.528) as the result of underestimation of the values in the former ELISA. At 24 wk, ETN concentrations were similar in patients with ASDAS < 2.1 and ≥ 2.1. Anti-ETN Abs were not detected in any of the patient samples tested. In conclusion, highly sensitive mAb-based immunoassays were developed for quantification of ETN and anti-ETN concentrations. The impact of these methods needs to be evaluated further in clinical practice. *The Journal of Immunology*, **2016, 196: 2879–2884.**

Ankylosing spondylitis (AS), the most common form of spondyloarthritis, is characterized by chronic inflammation of the spine and the sacroiliac joints. However, in a substantial number of patients, peripheral arthritis and different extra-articular manifestations add to the burden of disease (1). Until recently, the therapeutic options for AS have been limited to nonsteroidal anti-inflammatory drugs and physiotherapy because conventional disease-modifying anti-rheumatic drugs showed no or inconsistent effects. In contrast, the introduction of biological drugs heralded a new era for the treatment of AS (1, 2). Etanercept (ETN; Enbrel) is a fully human dimeric fusion protein that specifically binds to TNF, thereby modulating its biologic function and preventing its proinflammatory activities (3). Despite its proven efficacy in AS, the clinical response to treatment varies (4).

Currently, anti-TNF drugs are dosed using a one-size-fits-all approach, whereby patients receive the same or similar dose regimen, regardless of any variables that might affect the pharmacokinetic profile of the drug (5). Nonetheless, inadequate anti-TNF drug concentrations and the presence of antidrug Abs (ADAbs) frequently have been implicated as predisposing factors for therapeutic failure and side effects (6). Furthermore, a recent study by Kneepkens et al. (4) provided evidence for a significant association among disease activity, inflammation, and ETN concentrations in patients with AS at 24 wk of treatment.

However, therapeutic drug monitoring (TDM) of anti-TNF therapy has not been used in routine clinical practice of AS. This lack of implementation may be explained, in part, by discrepancies between the existing TDM platforms, which hamper the interpretation of drug concentrations and highlights the need for standardization. In addition, the lack of a "gold standard" for ADAb measurements makes comparison between different assays even more complex.

The main objective of this work was to develop highly sensitive immunoassays for the specific quantification of serum ETN and anti-ETN concentrations by taking advantage of a newly generated and extensively characterized panel of mouse mAbs. The clinical significance of the assays was additionally evaluated using serum samples from 21 AS patients receiving ETN maintenance dosing (50 mg/wk).

Materials and Methods

BSA, HRP, H_2SO_4, and citric acid were purchased from Sigma-Aldrich (Steinheim, Germany). VWR International (Haasrode, Belgium) delivered $Na_2HPO_4{\cdot}2H_2O$. KH_2PO_4 and Sulfo-NHS-LC-Biotin were obtained from Thermo Fisher Scientific (Waltham, MA). HRP-conjugated streptavidin was purchased from IBL International (Hamburg, Germany), and recombinant human TNF and sTNF receptor type II were from PeproTech (London, U.K.). H_2O_2 was obtained from Merck (Darmstadt, Germany), and *o*-phenylenediamine was from Acros Organics (Geel, Belgium). ETN was purchased as Enbrel from Pfizer SA/NV (Elsene, Belgium).

*Department of Pharmaceutical and Pharmacological Sciences, Laboratory for Therapeutic and Diagnostic Antibodies, KU Leuven, B-3000 Leuven, Belgium; †PharmAbs, The KU Leuven Antibody Center, B-3000 Leuven, Belgium; and ‡Department of Rheumatology, University Hospitals Leuven, B-3000 Leuven, Belgium

Received for publication October 9, 2015. Accepted for publication January 4, 2016.

This work was supported by the Fund for Scientific Research Flanders (Grant G. 0617.12), Investigator Initiated Research Grant WS2403121 from Pfizer, and the Agency for the Promotion and Innovation through Science and Technology in Flanders (to I.D.).

Address correspondence and reprint requests to Prof. Ann Gils, Laboratory for Therapeutic and Diagnostic Antibodies, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, O&N II, Herestraat 49, Box 820, B-3000 Leuven, Belgium. E-mail address: ann.gils@pharm.kuleuven.be

Abbreviations used in this article: ADAb, antidrug Ab; AS, ankylosing spondylitis; ASDAS, AS Disease Activity Score; CBA, cell-based assay; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ETN, etanercept; IQR, interquartile range; MA-ETN, mAb against ETN; QC, quality control; TDM, therapeutic drug monitoring.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1502195

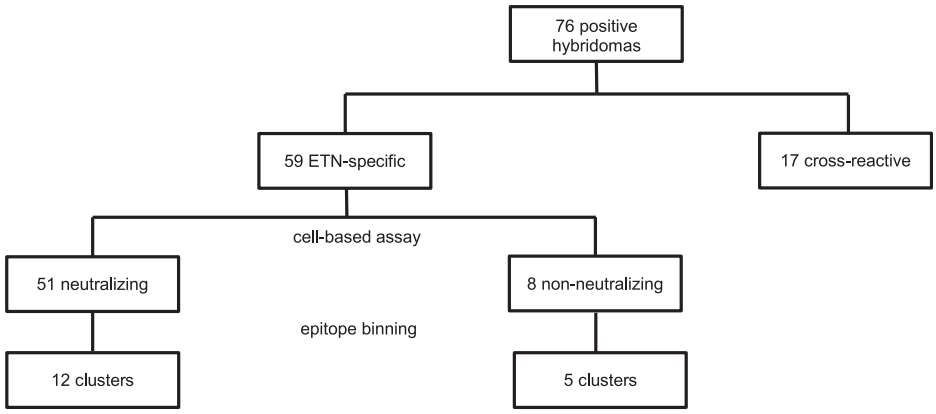

FIGURE 1. Flow chart of the generation and characterization of MA-ETN.

Patients

Serum samples were collected from 21 patients with AS under ETN maintenance therapy (50 mg ETN s.c. once a week) at the Department of Rheumatology, University Hospitals Leuven - campus Gasthuisberg. The samples were taken at their first hospital visit and at 24 wk of follow-up, without reference to the timing of the administration of their therapy (i.e., trough/nontrough not specified). Three of these patients administered the drug themselves before the first sample was taken; the other patients were anti-TNF naive. Disease activity was assessed at baseline and after 6 mo of anti-TNF therapy using the AS Disease Activity Score (ASDAS) (7). Active disease was defined as ASDAS $\geq$ 2.1 (8). The study was approved by the Ethical Committee UZ Leuven (S51013/S55065). Informed consent was provided by all patients.

mAb generation

Hybridomas producing mAbs were generated in-house upon immunization of SJL/J mice with ETN. The details of this protocol were described by Van Stappen et al. (9). Positive clones were selected, after which the cross-reactivity of the MA-ETN toward infliximab (Remicade), adalimumab (Humira), golimumab (Simponi), and a human IgG mixture (Multigam) was determined. The hybridomas that reacted exclusively with ETN were grown in Integra CELLine systems (Wheaton Industries, Millville, NJ) for large-scale production. The MA-ETN were purified from conditioned medium on a ProSep-vA Ultra column (Millipore, Billerica, MA) and conjugated with HRP.

Characterization of mAbs directed toward ETN

Neutralizing capacity. The neutralizing capacity of the mAbs against ETN (MA-ETN) was assessed in a cell-based assay (CBA). HT1080 (American Type Culture Collection) is a human fibrosarcoma cell line that expresses IL-6 upon stimulation with TNF. The TNF-inducible IL-6 secretion is inhibited by ETN. A neutralizing Ab can upregulate IL-6 expression by blocking the inhibitory effect of ETN on TNF. We described the details of this assay previously (10). MA-ETN, used in a 10-fold molar excess (150 ng/ml) over ETN, were added to sera supplemented with TNF (7.5 ng/ml, based on the TNF $EC_{50/80}$) and ETN (15 ng/ml, based on the ETN $IC_{50/80}$) after which the effect on IL-6 expression was determined. An MA-ETN was considered to be neutralizing when $\geq$80% of the initial IL-6 response could be retrieved.

Epitope binning. Epitope binning was carried out using competition ELISA (9). The MA-ETN ("cold" Abs) were tested against all HRP-conjugated MA-ETN ("labeled" Abs) in a pairwise combinatorial manner to determine region binding areas recognized by the two Abs relative to each other. For this, ETN was coated on the plate (at a concentration of 4 μg/ml), and the MA-ETN were added in excess (10 μg/ml) to the wells before the HRP-conjugated MA-ETN (0.025 μg/ml) were applied. The defined epitope regions were considered to be independent when the OD obtained for the labeled Ab in the presence (+) of cold Ab was $\geq$80% of its OD obtained in the absence (−) of cold Ab.

Affinity determination. Surface plasmon resonance–based affinity measurements of the selected MA-ETN were carried out using a Biacore 3000 (GE Healthcare, Uppsala, Sweden). Briefly, CM5 sensor chips were immobilized to ± 50 resonance units with ETN using *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride amine coupling chemistry. Then, the MA-ETN were applied at five concentrations between 50 and 1000 nM at a flow rate of 60 μl/min. Analysis of the association and dissociation rates was performed using BIAevaluation software (1:1 Langmuir binding global fit isotherm).

Isotyping. The isotypes of the selected MA-ETN were determined using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics, Vilvoorde, Belgium), according to the manufacturer's protocol.

Determination of serum concentrations of ETN

TNF-coated ELISA. Measurement of ETN serum concentrations was initially carried out by means of a TNF-coated ELISA setup, which, in principle, is similar to the test that is routinely used for quantification of infliximab concentrations (9). A newly generated and extensively characterized MA-ETN-HRP was used as conjugate. Performance characteristic of the ETN dose-response curve in the range of 0.8–50.0 ng/ml was evaluated by calculating the recovery percentage of three quality control (QC) samples (high, mid, and low) in buffer. Acceptance criterion was defined as an absolute mean percentage deviation $\leq$ 20% for each QC sample. The cut-off of the assay was set to have an upper negative limit of 99.7%, determined using the mean (+ 3 SD) OD of the anti-TNF naive AS patient samples (200-fold diluted).

Sandwich-type ELISA. ETN concentrations were also determined in a newly developed sandwich-type ELISA, based on the principle that ETN is captured between an immobilized MA-ETN and an added HRP-labeled MA-ETN that targets a different epitope on the ETN molecule. Briefly, 96-well plates were precoated with an MA-ETN and blocked with 1% BSA in PBS (pH 7.4) to minimize nonspecific protein binding. After washing, serum samples (1000-fold diluted) were added, and the plates were incubated overnight at 4°C. Bound ETN was subsequently detected by HRP-labeled MA-ETN. Plates were developed using o-phenylenediamine and H_2O_2 in sodium citrate, disodium phosphate buffer (pH 5), and the reaction was stopped with H_2SO_4. The absorbance was measured at 490 nm with an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski,

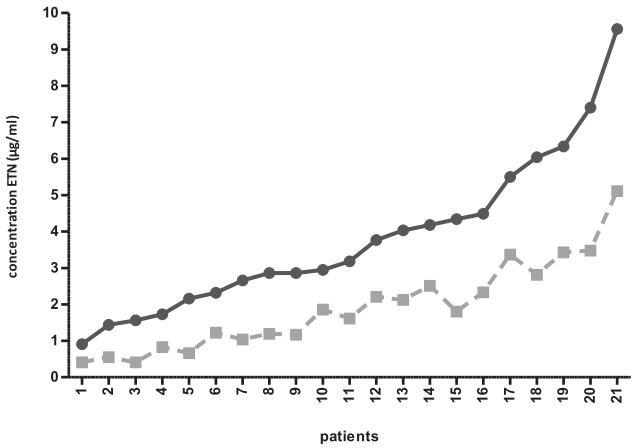

FIGURE 2. ETN concentrations of the 21 AS patients at 24 wk of treatment. ETN concentrations were measured with the mAb-based TNF-coated ELISA (dashed gray line) and the mAb-based sandwich-type ELISA (solid black line). All 21 patients were stratified according to ETN concentration (low to high) in the sandwich-type ELISA, with each data point representing the mean ETN concentration (n = 3).

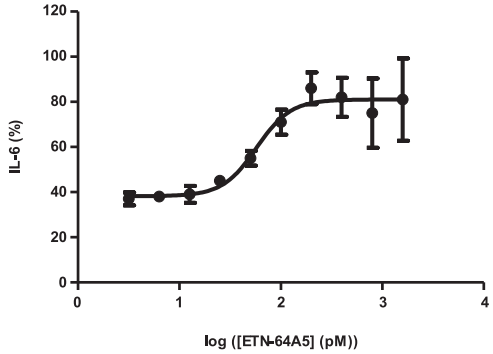

FIGURE 3. Dose-response curve of MA-ETN64A5. To determine the inhibitory effect of MA-ETN64A5, different concentrations (0–240 ng/ml) of MA-ETN64A5 were added to sera supplemented with 7.5 ng/ml TNF and 15 ng/ml ETN. Data are mean ± SD (n = 3).

VT), and the results were compared with a dose-response curve of ETN (0.2–10.0 ng/ml concentration range), present on each plate. Standard curve performance and cut-off of the assay were worked out analogously to the TNF-coated ELISA.

Assessment of MA-ETN

Bridging ELISA. The panel of in-house developed MA-ETN was further evaluated as calibrator in a bridging ELISA setup for quantification of the ADAb response toward ETN. This immunoassay depends on the bivalency of the MA-ETN and, hence, their ability to "bridge" an ETN molecule preabsorbed to a plastic plate with an added biotin-labeled ETN molecule. The use of biotin offered the advantage of a signal-amplification step when bound by HRP-conjugated streptavidin and o-phenylenediamine, sequentially applied on the 96-well plate (11). The MA-ETN calibration curve was applied in the range of 0.2–10.0 ng/ml, and three QC samples (high, mid, and low) were prepared in buffer to test its performance. The cut-off of the assay was established using the anti-TNF naive AS patient samples in a 20-fold dilution.

Cell-based assay. The same calibrator of the bridging ELISA was additionally evaluated in the in-house developed functional CBA to quantify the neutralizing Ab responses elicited to ETN. Although this technique is labor intensive and shows a large interassay variability (10), it has the advantage that both low- and high-affinity neutralizing Abs of different subtypes (including IgG4) can be measured. To determine the half-maximal inhibitory concentration (IC_{50}) of the calibrator, serial concentrations (0–240 ng/ml) were added to sera supplemented with 7.5 ng/ml TNF and 15 ng/ml ETN. The cut-off value of the CBA was calculated by using the anti-TNF naive AS patient samples in a 40-fold dilution under the same conditions as described above.

Statistical analyses

Quantitative data were summarized as mean and SD or median and interquartile range (IQR), as appropriate. The Shapiro–Wilk normality test was used to assess the normality of continuous variables. Univariate statistical analyses were performed using GraphPad Prism 5 (GraphPad, San Diego, CA). The paired t test and Wilcoxon matched-pairs signed-rank test were used to compare paired data. The unpaired t test and the Mann–Whitney U test were used to compare unpaired data. The Pearson correlation test was used to analyze correlation. A two-sided p value < 0.05 was considered statistically significant.

Results

Generation and characterization of the MA-ETN

Four fusions of Sp2/0-Ag14 myeloma cells with spleen cells isolated from immunized SJL/J mice yielded a total of 76 positive hybridoma clones producing mAbs toward ETN (Fig. 1). Of these Abs, 78% (59/76) showed no (cross-)reactivity with any of the other anti-TNF drugs nor with the human IgG mixture and were considered ETN specific. The 59 ETN-specific mAbs were successfully purified from conditioned medium and subjected to an in-depth characterization (Fig. 1). A first evaluation of their neutralizing capacity revealed that the majority (51 of 59, 86%) strongly inhibited the TNF–ETN interaction. For the remaining eight (14%) MA-ETN, no inhibitory effect was observed (Fig. 1). The 51 neutralizing MA-ETN were further assigned to 12 distinct clusters based upon their binning profiles. The MA-ETN with no inhibitory properties eventually fell into five unique epitope bins (Fig. 1).

Determination of serum concentrations of ETN

TNF-coated and sandwich-type assay performance validation. Fifty-nine MA-ETN belonging to 17 clusters were tested for their suitability as conjugate in the construction of a TNF-coated ELISA for quantification of ETN serum concentrations. Based upon the sensitivity and specificity of the obtained response, only the noninhibitory MA-ETN68C5-HRP proved to be useful for further evaluation of the assay. The ETN calibration curve was nonlinear between 0.8 and 50.0 ng/ml, and the assay cut-off was set at 0.2 μg/ml (taking into account a serum dilution factor of 1:200). The three QC samples, final concentrations of 40, 20, and 10 ng/ml, yielded recoveries of 89 ± 5, 85 ± 6, and 67 ± 3%, respectively (mean ± SD, n = 3). For the construction of the sandwich-type ELISA, a similar approach was used testing 17 MA-ETN × 17 MA-ETN-HRP combinations to capture and detect ETN in this setup. A high-affinity compatible pair of Abs (MA-ETN63C8/MA-ETN61C1-HRP) was selected for development of the assay. Selection was based on their strict specificity for ETN and on their important neutralizing activity. Using this combination, a linear dose-response curve was obtained in the range of 0.2–10.0 ng/ml. The assay cut-off was set at 0.2 μg/ml (taking into account a serum dilution factor of 1:1000). The recoveries of the three QC samples, final concentrations of 8, 4, and 2 ng/ml, were 94 ± 4, 95 ± 4, and 95 ± 6%, respectively (mean ± SD, n = 3), indicating that the sandwich-type ELISA is accurate and closely reflects the amount of ETN present in the sample.

Comparative analysis of 21 AS patient samples at 24 wk of follow-up. A significant and high correlation was observed between the two in-house–developed ELISA methods (sandwich versus TNF coated) for measuring serum ETN concentrations (r = +0.974, $p < 0.0001$). Nevertheless, mean ETN concentration was considerably higher in the sandwich-type ELISA (3.8 μg/ml, SD 2.2 μg/ml) compared with the TNF-coated ELISA (1.9 μg/ml, SD 1.2 μg/ml) ($p < 0.0001$). Fig. 2 shows the ETN concentrations (low to high) in the 21 patients with AS at 24 wk of treatment. Based upon an intraclass correlation coefficient of 0.528, the methods cannot be considered interchangeable (12).

Extensive evaluation of the newly developed sandwich-type ELISA. To confirm that ETN concentrations were not markedly overestimated in the sandwich-type ELISA and to further evaluate

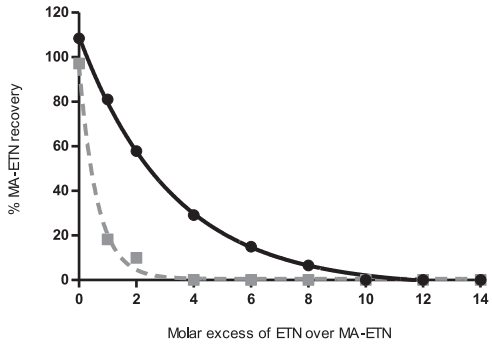

FIGURE 4. Drug-interference effect in the bridging ELISA. Percentage recovery of 80 ng/ml MA-ETN64A5 (solid black line) and 2000 ng/ml MA-ETN68C5 (dashed gray line) after incubation with a titration of ETN (n = 2).

Table I. Disease status of the 21 AS patients at baseline and at 24 wk of therapy

Variables	Baseline	24 Wk
BASDAI (Q2, Q3, Q6)	7.0 (5.5–8.0)	3.0 (1.0–4.0)*
PG DAS	7.0 (4.5–8.0)	2.0 (1.0–5.0)*
CRP	11.7 (4.5–20.6)	1.3 (0.6–7.4)*
ESR	27.0 (21.5–47.0)	10.0 (4.0–20.0)*

Data are expressed as median (IQR). Normal CRP < 3.0 mg/l; normal ESR < 10 mm/h.

*$p < 0.05$.

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index (0–10): Back pain, BASDAI Q2; peripheral pain/swelling, BASDAI Q3; duration of morning stiffness, BASDAI Q6; PG DAS, Patient Global Disease Activity Score (0–10).

the performance of the assay, some additional experiments were conducted. Effective prevention of nonspecific binding was assessed by means of a competition experiment using a 6-fold molar excess of MA-ETN61C1 (the conjugate Ab of the assay) over ETN. For this purpose, a patient sample with a measured ETN concentration of 1 μg/ml was selected. The sample was incubated for 20 min at 37°C in the presence and absence of 6 μg/ml MA-ETN61C1, followed by measurement of the concentration in the sandwich-type ELISA. Addition of 6 μg/ml MA-ETN61C1 revealed a response below the cut-off of the assay (data not shown). Accuracy and imprecision of the assay were determined by analyzing seven replicates of each QC sample in triplicate. The experiments were performed in buffer and in patient sera. All QC samples reached the minimum acceptable criteria: an absolute mean percentage deviation $\leq 20\%$ (for accuracy) and a coefficient of variation $\leq 15\%$ (for imprecision). To exclude interference of endogenous sTNF receptor type II, serial 2-fold dilutions of 50 ng/ml recombinant sTNF receptor type II were applied on the MA-ETN63C8/MA-ETN61C1-HRP combination, revealing a 100-fold lower reactivity compared with ETN, with a reactivity similar to background from 6.25 ng/ml onward (data not shown).

Assessment of MA-ETN

Bridging and CBA performance validation. Of all MA-ETN that were able to cross-link coated ETN with biotin-labeled ETN, MA-ETN64A5 was selected as calibrator in the bridging ELISA. MA-ETN64A5 is a high-affinity and inhibitory IgG1 anti-ETN Ab with κ L chains that specifically binds to ETN. With the use of MA-ETN64A5 as calibrator, a linear dose-response curve was obtained in the range of 0.2–10.0 ng/ml. The cut-off of the assay was set at 3.2 ng/ml equivalents MA-ETN64A5 (taking into account a serum dilution factor of 1:20), and its performance was established by the recoveries of the three QC samples (data not shown). Assessment of the inhibitory effect of MA-ETN64A5 in the CBA revealed an IC_{50} of 10.8 ng/ml upon addition of 7.5 ng/ml TNF and 15 ng/ml ETN (Fig. 3). Using the anti-TNF naive AS patient samples, the cut-off in the CBA was subsequently set at 200 ng/ml equivalents MA-ETN64A5 (taking into account a serum dilution factor of 1:40).

Comparative analysis of 21 AS patient samples at 24 wk of follow-up. Patients' sera were first analyzed in the bridging ELISA. However, no anti-ETN Abs were detected. Because the maintenance regimen for AS patients treated with ETN requires weekly dosing, collection of patient samples with undetectable serum ETN concentrations is a challenge. To ensure reliable assessment in the CBA, the anti-TNF naive patient samples and their corresponding follow-up samples (all containing measurable ETN concentrations) were applied to the HT1080 cells. Basal IL-6 concentrations were 1.4 ± 0.5 ng/ml (mean ± SD). Upon addition of 7.5 ng/ml TNF to individual serum samples, the increased IL-6 production was set at 100% for each anti-TNF naive patient sample, and IL-6 production of the follow-up sample was calculated versus this 100%. For 94% (17/18) of the samples, addition of the follow-up sample revealed an IL-6 response that was 69 ± 18% (mean ± SD) lower than the response of its naive sample, indicating the presence of functionally active ETN. When an additional 15 ng/ml ETN was added to the sera, IL-6 production decreased to background concentrations in all patient samples (data not shown).

Extensive evaluation of the bridging ELISA. The detection of ADAbs is affected by excess of the drug in the sample itself. To test the performance of the bridging ELISA in the presence of serum ETN, a noninhibitory (MA-ETN68C5) and an inhibitory (MA-ETN64A5) anti-ETN mAb were selected and preincubated with graded concentrations of ETN, and the reduction in signal was evaluated. The noninhibitory MA-ETN68C5 demonstrated a lower affinity for ETN ($K_A = 3.2 \times 10^7\ M^{-1}$) compared with MA-ETN64A5 ($K_A = 7.7 \times 10^8\ M^{-1}$), and a concentration of 2000 ng/ml was required to give a signal above the cut-off in the bridging ELISA. As shown in Fig. 4, a 10-fold molar excess of ETN over both MA-ETN completely abolished the binding and resulted in undetectable MA-ETN concentrations. Accuracy and imprecision of the assay were determined analogously to the sandwich-type ELISA. All QC samples reached the minimum acceptable criteria (data not shown).

Analysis of AS patient samples

A total of 21 AS patients with an ASDAS of 3.8 ± 1.2 (mean ± SD) was included in this study. The baseline disease status of these patients is shown in Table I. Treatment with ETN resulted in general improved outcomes compared with baseline (ASDAS of 1.8 ± 1.3). Nevertheless, at 24 wk of therapy, 8 of the 21 ETN-treated AS patients had a high disease activity indicated by ASDAS ≥ 2.1. As shown in Table II, there was no significant difference in baseline clinical and biochemical characteristics between patients with ASDAS ≥ 2.1 (at 24 wk) and patients with ASDAS < 2.1 (at 24 wk). At week 24, the mean ETN

Table II. Clinical and biochemical characteristics for patients with ASDAS < 2.1 ($n = 13$) and ASDAS ≥ 2.1 ($n = 8$)

	ASDAS < 2.1 (at 24 wk)		ASDAS ≥ 2.1 (at 24 wk)	
Variables	Baseline	24 Wk	Baseline	24 Wk
BASDAI (Q2, Q3, Q6; median [IQR])	7.0 (4.5–8.0)	1.0 (0.5–3.0)*	7.0 (6.0–9.5)	4.0 (3.3–7.5)*
PG DAS (mean [SD])	6.0 (2.6)	1.8 (1.4)*	7.0 (1.3)	5.1 (2.7)*
CRP (median [IQR])	11.7 (2.1–28.0)	0.6 (0.2–1.3)*	11.6 (10.3–19.4)	9.7 (3.0–23.5)
ESR (median [IQR])	26.0 (18.0–68.5)	6.0 (3.0–13.5)*	36.0 (26.0–45.0)	20.0 (11.5–36.3)
ETN (μg/ml; mean [SD])[a]		3.8 (2.5)		3.9 (1.6)

[a]Measured with the mAb-based sandwich-type ELISA.

*$p < 0.05$.

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index (0–10): Back pain, BASDAI Q2; peripheral pain/swelling, BASDAI Q3; duration of morning stiffness, BASDAI Q6; PG DAS, Patient Global Disease Activity Score (0–10).

concentration was similar for both groups. However, for patients with ASDAS $\geq$ 2.1, the two nonpatient-reported measurements, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were not significantly improved from baseline (Table II).

Discussion

The importance of TDM of anti-TNF agents has attracted considerable attention in recent years. Several clinical studies strongly suggest that a personalized approach to chronic disease management, in which therapeutic decisions are not based solely on assessment of clinical symptoms but also on drug and ADAb determinations, will result in safer and more (cost-)effective patient care (13–15). Nonetheless, conflicting reports originating from the use of inadequately designed assays might complicate the reliability of TDM in everyday clinical practice of AS. The aim of the current study was to develop highly sensitive immunoassays for the specific quantification of serum ETN and anti-ETN concentrations by taking advantage of a newly generated and extensively characterized panel of mouse mAbs.

For the measurement of ETN serum concentrations, two ELISA types were developed. Performance validation of the TNF/MA-ETN68C5-HRP ELISA yielded poor recoveries in a spiking experiment with three ETN concentrations. This is in contrast to the recovery experiments in the MA-ETN63C8/MA-ETN61C1-HRP ELISA for which good results were obtained. The reason for the underestimation in the TNF-coated ELISA is unclear, but it might be attributable to some nonspecific conformational changes in the TNF molecules immobilized on the 96-well plate. In this regard, it is commonly believed that ETN binding is restricted to the trimer form of TNF, with receptor binding site(s) in the cleft formed between two TNF subunits (16). It is possible that these binding sites became less accessible when TNF was coated on the plate. In contrast, for the anti-TNF mAbs (e.g., golimumab), a high intraclass correlation coefficient agreement was obtained between the respective ELISA types (17). However, these anti-TNF mAbs are known to bind both monomeric and trimeric forms of TNF (16). Applying the MA-ETN63C8/MA-ETN61C1-HRP ELISA, mean ETN concentration in the 21 AS patient samples was 3.8 μg/ml (SD 2.2 μg/ml) at 24 wk of treatment. This finding is more or less in line with previous studies in AS (4, 18–20), in which mean ETN concentrations of ∼3 μg/ml were reported at 24 wk of therapy. In these studies, ETN was also captured through its ability to bind TNF; however, the set-up differed slightly from the TNF-coated ELISA described in this article, because a specific mouse mAb was used to immobilize TNF on the plate. To exclude interference of endogenous sTNF receptor type II, recombinant sTNF receptor type II was applied on the ELISA combination, revealing a 100-fold lower response compared with ETN. Taking into account that the reported sTNF receptor type II concentration in AS patients is 2.8 ng/ml (21), and thus ∼1000-fold lower compared with the mean serum ETN concentration of 3.8 μg/ml (SD 2.2 μg/ml) in our cohort of AS patients, interference of endogenous sTNF receptor type II in the detection of ETN using the MA-ETN63C8/MA-ETN61C1-HRP ELISA can be ruled out.

The inhibitory MA-ETN64A5, generated in the study, was equally well suited to quantify the total amount of anti-ETN Abs in a bridging ELISA as to determine the proportion of neutralizing anti-ETN Abs in a functional CBA. However, anti-ETN Abs were not detected in any of the patient sera tested, which seems to support the hypothesis that ETN is not or only marginally immunogenic (18). Although there is a possibility that some positive results were missed as a result of interference of drug in the sample, the overall reported frequency of anti-ETN Abs is low (19). In each case in which anti-ETN Abs were detected, these Abs tended not to neutralize the drug's biological activity or compromise its long-term effectiveness (19, 20).

In contrast to Kneepkens et al. (4), we did not find any difference in ETN concentrations between patients with ASDAS $<$ 2.1 and patients with ASDAS $\geq$ 2.1 at 24 wk of therapy. However, for patients with ASDAS $\geq$ 2.1, the two objective parameters of inflammation, CRP and ESR, did not improve significantly from baseline. This is notable because ETN is specifically engineered to treat inflammation, and it raises the question of whether therapy should be discontinued in such patients because of lack of efficacy. Theoretically, there are many reasons for ETN nonresponsiveness, with a non-TNF–driven disease phenotype being one of the possibilities (22). This so-called "true" nonresponse to ETN therapy (despite adequate concentrations of drug) could have impaired the interpretation of the pharmacokinetic relationships between response and remission. Other factors, associated with low drug concentrations (e.g., patient-specific characteristics) (23), and, thus, an incomplete suppression of TNF activity (also referred to as a "partial" nonresponse), could not be assessed in this study. Moreover, given the small number of patients and the random timing of sampling, these data must be interpreted with caution.

In conclusion, highly sensitive mAb-based immunoassays were developed for the specific quantification of serum ETN and anti-ETN concentrations and applied to a cohort of 21 AS patients. However, further research is needed to evaluate the impact of these methods in clinical decision-making for AS patients when added to current practice.

Disclosures

A.G. has received grant support and speaker fees from Pfizer. The other authors have no financial conflicts of interest.

References

1. Vander Cruyssen, B., C. Ribbens, A. Boonen, H. Mielants, K. de Vlam, J. Lenaerts, S. Steinfeld, F. Van den Bosch, L. Dewulf, and N. Vastesaeger. 2007. The epidemiology of ankylosing spondylitis and the commencement of anti-TNF therapy in daily rheumatology practice. *Ann. Rheum. Dis.* 66: 1072–1077.
2. Gorman, J. D., K. E. Sack, and J. C. Davis, Jr. 2002. Treatment of ankylosing spondylitis by inhibition of tumor necrosis factor α. *N. Engl. J. Med.* 346: 1349–1356.
3. Goffe, B., and J. C. Cather. 2003. Etanercept: An overview. *J. Am. Acad. Dermatol.* 49(2, Suppl.): S105–S111.
4. Kneepkens, E. L., C. L. Krieckaert, D. van der Kleij, M. T. Nurmohamed, I. E. van der Horst-Bruinsma, T. Rispens, and G. J. Wolbink. 2015. Lower etanercept levels are associated with high disease activity in ankylosing spondylitis patients at 24 weeks of follow-up. *Ann. Rheum. Dis.* 74: 1825–1829.
5. Stamp, L. K., and M. Barclay. 2014. Therapeutic drug monitoring in rheumatic diseases: utile or futile? *Rheumatology (Oxford)* 53: 988–997.
6. Chaparro, M., I. Guerra, P. Muñoz-Linares, and J. P. Gisbert. 2012. Systematic review: antibodies and anti-TNF-α levels in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 35: 971–986.
7. van der Heijde, D., E. Lie, T. K. Kvien, J. Sieper, F. Van den Bosch, J. Listing, J. Braun, and R. Landewé, Assessment of SpondyloArthritis international Society (ASAS). 2009. ASDAS, a highly discriminatory ASAS-endorsed disease activity score in patients with ankylosing spondylitis. *Ann. Rheum. Dis.* 68: 1811–1818.
8. Machado, P., R. Landewé, E. Lie, T. K. Kvien, J. Braun, D. Baker, and D. van der Heijde, Assessment of SpondyloArthritis international Society. 2011. Ankylosing Spondylitis Disease Activity Score (ASDAS): defining cut-off values for disease activity states and improvement scores. *Ann. Rheum. Dis.* 70: 47–53.
9. Van Stappen, T., E. Brouwers, S. Tops, N. Geukens, S. Vermeire, P. J. Declerck, and A. Gils. 2015. Generation of a highly specific monoclonal anti-infliximab antibody for harmonization of TNF-coated infliximab assays. *Ther. Drug Monit.* 37: 479–485.
10. Gils, A., N. Vande Casteele, R. Poppe, M. Van de Wouwer, G. Compernolle, M. Peeters, E. Brouwers, S. Vermeire, N. Geukens, and P. J. Declerck. 2014. Development of a universal anti-adalimumab antibody standard for interlaboratory harmonization. *Ther. Drug Monit.* 36: 669–673.
11. Van Stappen, T., T. Billiet, N. Vande Casteele, G. Compernolle, E. Brouwers, S. Vermeire, and A. Gils. 2015. An Optimized Anti-infliximab Bridging Enzyme-linked Immunosorbent Assay for Harmonization of Anti-infliximab Antibody Titers in Patients with Inflammatory Bowel Diseases. *Inflamm. Bowel Dis.* 21: 2172–2177.

12. Vincent, W. J., and J. P. Weir. 1995. Quantifying reliability. In *Statistics in Kinesiology*, 4th Ed. W. J. Vincent and J. P. Weir, eds. Human Kinetics, Champaign, IL, p. 214–228.
13. Vande Casteele, N., M. Ferrante, G. Van Assche, V. Ballet, G. Compernolle, K. Van Steen, S. Simoens, P. Rutgeerts, A. Gils, and S. Vermeire. 2015. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. *Gastroenterology* 148: 1320–1329.e3.
14. Bendtzen, K., M. Ainsworth, C. Steenholdt, O. Ø. Thomsen, and J. Brynskov. 2009. Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. *Scand. J. Gastroenterol.* 44: 774–781.
15. Bendtzen, K., P. Geborek, M. Svenson, L. Larsson, M. C. Kapetanovic, and T. Saxne. 2006. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. *Arthritis Rheum.* 54: 3782–3789.
16. Scallon, B., A. Cai, N. Solowski, A. Rosenberg, X. Y. Song, D. Shealy, and C. Wagner. 2002. Binding and functional comparisons of two types of tumor necrosis factor antagonists. *J. Pharmacol. Exp. Ther.* 301: 418–426.
17. Detrez, I., E. Dreesen, T. Van Stappen, A. de Vries, E. Brouwers, G. Van Assche, S. Vermeire, M. Ferrante, and A. Gils. 2016. Variability in golimumab exposure: a 'real-life' observational study in active ulcerative colitis. *J. Crohns Colitis.* DOI: 10.1093/ecco-jcc/jjv241.
18. de Vries, M. K., I. E. van der Horst-Bruinsma, M. T. Nurmohamed, L. A. Aarden, S. O. Stapel, M. J. Peters, J. C. van Denderen, B. A. Dijkmans, and G. J. Wolbink. 2009. Immunogenicity does not influence treatment with etanercept in patients with ankylosing spondylitis. *Ann. Rheum. Dis.* 68: 531–535.
19. Vincent, F. B., E. F. Morand, K. Murphy, F. Mackay, X. Mariette, and C. Marcelli. 2013. Antidrug antibodies (ADAb) to tumour necrosis factor (TNF)-specific neutralising agents in chronic inflammatory diseases: a real issue, a clinical perspective. *Ann. Rheum. Dis.* 72: 165–178.
20. Arends, S., H. R. Lebbink, A. Spoorenberg, L. B. Bungener, C. Roozendaal, E. van der Veer, P. M. Houtman, E. N. Griep, P. C. Limburg, C. G. Kallenberg, et al. 2010. The formation of autoantibodies and antibodies to TNF-α blocking agents in relation to clinical response in patients with ankylosing spondylitis. *Clin. Exp. Rheumatol.* 28: 661–668.
21. Schulz, M., H. Dotzlaw, and G. Neeck. 2014. Ankylosing spondylitis and rheumatoid arthritis: serum levels of TNF-α and its soluble receptors during the course of therapy with etanercept and infliximab. *BioMed Res. Int.* 2014: 675108.
22. Papamichael, K., A. Gils, P. Rutgeerts, B. G. Levesque, S. Vermeire, W. J. Sandborn, and N. Vande Casteele. 2015. Role for therapeutic drug monitoring during induction therapy with TNF antagonists in IBD: evolution in the definition and management of primary nonresponse. *Inflamm. Bowel Dis.* 21: 182–197.
23. Zhou, H., M. Buckwalter, J. Boni, P. Mayer, D. Raible, J. Wajdula, S. Fatenejad, and M. Sanda. 2004. Population-based pharmacokinetics of the soluble TNFr etanercept: a clinical study in 43 patients with ankylosing spondylitis compared with post hoc data from patients with rheumatoid arthritis. *Int. J. Clin. Pharmacol. Ther.* 42: 267–276.